The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Dated 21 August 2003

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patents Form 1/77

Patents Act 1977
(Rule 16)

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office
Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — MJL/KMM/B45338

2. Patent application number *(The Patent Office will fill in his part)* — 0230170.3 — 24 DEC 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

   GlaxoSmithKline Biologicals s.a.
   Rue de l'Institut 89, B-1330 Rixensart, , Belgium

   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — Belgian — 08530586001

4. Title of the invention — vaccine

5. Name of your agent *(if you have one)*

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

   Patents ADP number *(if you know it)*

   Corporate Intellectual Property

   GlaxoSmithKline
   Corporate Intellectual Property (CN9 25.1)
   980 Great West Road
   BRENTFORD
   Middlesex TW8 9GS — 07960982003

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right

to grant of a patent required in support of this request? *(Answer yes if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is named as an applicant, or
c) any named applicant is a corporate body
See note (d)

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	23
Description	6
Claim(s)	1
Abstract	1
Drawings	1

10. If you are also filing any of the following, state how many against each item.

Priority Documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. We request the grant of a patent on the basis of this application
Signature Michael Lubienski Date 24-Dec-02
M J Lubienski

12. Name and daytime telephone number of person to contact in the United Kingdom: M J Lubienski 020 80474434

Warning

After an application for a Patent has beeen filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission unless an application has been filed at least six weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505*
b) *Write your answers in capital letters using black ink or you may type them.*
c) *If there is not enough space for all relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be*

attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

f) For details of the fee and ways to pay please contact the Patent Office.

Patents Form 1/77

VACCINE

FIELD OF THE INVENTION

The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are more suitable for the production of neisserial, in particular meningococcal, outer-membrane vesicle (or bleb) vaccines. Advantageous processes and vaccine products are also described based on the use of novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines which have been rendered safer and more effective for use in human subjects.

BACKGROUND OF THE INVENTION

Neisseria meningitidis (meningococcus) is a Gram negative bacterium frequently isolated from the human upper respiratory tract. It is a cause of serious invasive bacterial diseases such as bacteremia and meningitis. The incidence of meningococcal disease shows geographical, seasonal and annual differences (Schwartz, B., Moore, P.S., Broome, C.V.; Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). The bacterium is commonly classified according to the serogroup if its capsular polysaccharide.

Most disease in temperate countries is due to strains of serogroup B and varies in incidence from 1-10/100,000/year total population - sometimes reaching higher values (Kaczmarski, E.B. (1997), Commun. Dis. Rep. Rev. 7: R55-9, 1995; Scholten, R.J.P.M., Bijlmer, H.A., Poolman, J.T. et al. Clin. Infect. Dis. 16: 237-246, 1993; Cruz, C., Pavez, G., Aguilar, E., et al. Epidemiol. Infect. 105: 119-126, 1990).

Epidemics dominated by serogroup A meningococci, mostly in central Africa, sometimes reach incidence levels of up to 1000/100,000/year (Schwartz, B., Moore, P.S., Broome, C.V. Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). Nearly all cases as a whole of meningococcal disease are caused by serogroup A, B, C, W-135 and Y meningococci, and a tetravalent A, C, W-135, Y capsular polysaccharide vaccine is available (Armand, J., Arminjon, F., Mynard, M.C., Lafaix, C., J. Biol. Stand. 10: 335-339, 1982).

The frequency of *Neisseria meningitidis* infections has risen in the past few decades in many European countries. This has been attributed to increased

transmission due to an increase in social activities (for instance swimming pools, theatres, etc.). It is no longer uncommon to isolate *Neisseria meningitidis* strains that are less sensitive or resistant to some of the standard antibiotics. This phenomenon has created an unmet medical need and demand for new anti-microbial agents, vaccines, drug screening methods, and diagnostic tests for this organism.

The available polysaccharide vaccines are currently being improved by way of chemically conjugating them to carrier proteins (Lieberman, J.M., Chiu, S.S., Wong, V.K., et al. JAMA 275 : 1499-1503, 1996).

A serogroup B vaccine, however, is not available. The serogroup B capsular polysaccharide has been found to be nonimmunogenic - most likely because it shares structural similarity with host components (Wyle, F.A., Artenstein, M.S., Brandt, M.L. et al. J. Infect. Dis. 126: 514-522, 1972; Finne, J.M., Leinonen, M., Mäkelä, P.M. Lancet ii.: 355-357, 1983). Effort has therefore been focused in trying to develop serogroup B vaccines from outer membrane vesicles (or blebs) or purified protein components therefrom.

Alternative meningococcal antigens for vaccine development are meningococcal lipooligosaccharides (LOS). These are outer membrane bound glycolipids which differ from the lipopolysaccharides (LPS) of the Enterobacteriaceae by lacking the O side chains, and thus resemble the rough form of LPS (Griffiss et al. Rev Infect Dis 1988; 10: S287-295). Heterogeneity within the oligosaccharide moiety of the LOS generates structural and antigenic diversity among different meningococcal strains (Griffiss et al. Inf. Immun. 1987; 55: 1792-1800). This has been used to subdivide the strains into 12 immunotypes. Immunotypes L3, L7, L9 have an identical carbohydrate structure and have therefore been designated L3,7,9 (or, for the purposes of this specification, generically as "L3"). Meningococcal LOS L3,7,9 (L3), L2 and L5 can be modified by sialylation, or by the addition of cytidine 5'-monophosphate-N-acetylneuraminic acid. Antibodies to LOS have been shown to protect in experimental rats against infection and to contribute to the bactericidal activity in children infected with *N. meningitidis* (Griffiss et al J Infect Dis 1984; 150: 71-79).

A problem associated with the use of LOS in a meningococcal vaccine, however, is its toxicity (due to its Lipid A moiety).

LOS is also present on the surface of meningococcal blebs. For many years efforts have been focused on developing meningococcal outer membrane vesicle (or

bleb) based vaccines (de Moraes, J.C., Perkins, B., Camargo, M.C. et al. Lancet 340: 1074-1078, 1992; Bjune, G., Hoiby, E.A. Gronnesby, J.K. et al. 338: 1093-1096, 1991). Such vaccines have the advantage of including several integral outer-membrane proteins in a properly folded conformation which can elicit a protective immunological response when administered to a host. In addition, Neisserial strains (including *N. meningitidis* serogroup B - menB) excrete outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. More often, however, blebs are prepared by methods comprising a detergent (e.g. deoxycholate) extraction of the bacterial cells (e.g. EP 11243), which has the effect of removing a lot of the LOS from the vaccine. This is desired due to the toxicity of LOS (also called endotoxin) as described above.

A further problem with using LOS as a vaccine antigen is that 12 LPS immunotypes exist with a diverse range of carbohydrate-structures (M. P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021). Antibodies raised against one immunotype fail to recognise a different immunotype. Although effort has been focused on producing a generic "core" region of the oligosaccharide portions of the LOS immunotypes (e.g. WO 94/08021), the bactericidal activity of antibodies generated against the modified LOS is lost. Thus a vaccine may need to have many LOS components of different immunotype to be effective.

A further problem exists with the use of LOS (also known as LPS or lipopolysaccharide) as antigens in human vaccines, namely that they carry saccharide structures that are similar to human sacharide structures, thus posing a safety issue with their use.

The present invention presents processes for ameliorating one or more of the above problems, and presents methods for making novel vaccines based on meningococcal LOS as a protective antigen, particularly when present on an outer membrane vesicle.

DESCRIPTION OF THE INVENTION

The subject matter of and information disclosed within the publications and patents or patent applications mentioned in this specification are incorporated by reference herein.

Reference to "lipooligosaccharide" (or "LOS") may also be referred to as "lipopolysaccharide" or "LPS".

The present inventors have found that the shortening LOS oligosaccharide structures leads to the loss of epitopes that can elicit a bacteriocidal immune response. Instead, the inventors have found that in order to use LOS most effectively in a vaccine formuation, the LOS oligosaccharide structure must be retained as much as possible, but a combination of just 2 LOS antigens can yield a universally effective Neisserial (preferably meningococcal) vaccine. A first aspect of the invention is an immunogenic composition for the prevention or treatment of Neisserial (preferably meningococcal or meningococcal B) disease comprising Neisserial (preferably meningococcal) LOS of immunotype L2 and LOS of immunotype L3. LOS may be isolated by either known purification procedures, or may be present in at least 2 outer membrane vesicle (or bleb) preparations derived from L2 and L3 Neisserial strains. In order to remove toxic loosly held LOS from the bleb preparation, but retain high levels of integrated LOS antigen in the bleb, it is preferred that the blebs are extracted using a low concentration of detergent – 0-0.3%, preferably 0.05-0.2%, most preferably around 0.1%, preferably deoxycholate (or DOC). Such a combination of LOS antigens is surprisingly advantageous in being effective against over 90% of *N meningitidis* strains.

The inventors have also found that the above bleb immunogenic compositions of the invention, and indeed any Neisserial (preferably gonococcal or meningococcal) derived bleb immunogenic composition, can have enhanced effect of protective antigens (including LOS) on their surface if certain combinations of immunodominant outer membrane proteins are downregulated in expression (and preferably deleted). A second aspect of the invention is therefore a Neisserial bleb preparation derived from a neisserial strain which has had 2 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native, non-modified strain: PorA, PorB, OpA, OpC or PilC. Preferably PorA and OpA, PorA and OpC, OpA and OpC, or PorA & OpA & and OpC are downregulated or deleted. Such mutations are beneficial in any Neisserial (preferably meningococcal) strain from which bleb immunogenic compositions are to be derived, however it is preferred that L2 or L3 immuntype Neisserial (preferably meningococcal) strains are used. Preferably the bleb immunogenic compositions of the invention contains both L2 and

L3 blebs where at least one (and preferably both) is deficient in the above combinations of immunodominant outer membrane proteins (or OMPs). Techniques for downregulating these genes are discussed in WO 01/09350 (incorporated by reference herein). Four different Opa genes are known to exist in the meningococcal genome (Aho et al. 1991 Mol. Microbiol. 5:1429-37), therefore where Opa is said to be downregulated in expression it is meant that preferably 1, 2, 3 or (preferably) all 4 genes present in meningococcus are so downregulated. Such downregulation may be performed genetically as described in WO 01/09350 or by seeking readily-found, natural, stable meningococcal strains that have no or low expression from the Opa loci. Such strains can be found using the technique described in Poolman et al (1985 J. Med. Micro. 19:203-209) where cells that are Opa$^-$ have a different phenotype to cells expressing Opa which can be seen looking at the appearance of the cells on plates or under a microscope. Once found, the strain can be shown to be stably Opa$^-$ by performing a Western blot on cell contents after a fermentation run to establish the lack of Opa.

Safety of the above LPS immunogenic compositions

The safety of antibodies raised to L3 or L2 LPS has been questioned, due to the presence of a structure similar to the lacto-N-neotetraose oligosaccharide group present in human glycosphingolipids. Even if a large number of people has been safely vaccinated with deoxycholate extracted vesicle vaccines containing residual amount of L3 LPS (G. Bjune *et al*, Lancet (1991), **338**, 1093-1096; GVG. Sierra *et al*, NIPH ann (1991), **14**, 195-210), the deletion of the terminal part of the LOS saccharidic is advantageous in preventing any cross-reaction with structures present at the surface of human tissues. Inactivation of the *lgtB* gene results in an intermediate LPS structure in which the terminal galactose residue and the sialic acid are absent (see figure 1 and 2). Such intermediates could be obtained in an L3 and an L2 LPS strain. An alternative and less preferred (short) version of the LPS can be obtained by turning off the *lgtE* gene. A further alternative and less preferred version of the LPS can be obtained by turning off the lgtA gene. If such an lgtA$^-$ mutation is selected it is preferred to also turn off lgtC expression to prevent the non-immunogenic L1 immunotype being formed.

LgtB$^-$ mutants are most preferred as the inventors have found that this is the optimal truncation for resolving the safety issue whilst still retaining an LPS protective oligosaccharide epitope that can still induce a bactericidal antibody response.

Therefore, the above L2 or L3 preparations (whether purified or in an isolated bleb) of the invention or meningococcal bleb preparations in general are advantageously derived from a Neisserial strain (preferably meningococcal) that has been genetic engineered to permanently downregulate the expression of functional gene product from the lgtB, lgtA or lgtE gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

Where the above bleb preparations of the invention are derived from a meningococcus B strain, it is further preferred that the capsular polysaccharide (which also contains human-like saccharide structures) is also removed. Although many genes could be switched off to achieve this, the inventors have advantageously shown that it is preferred that the bleb production strain has been genetically engineered to permanently downregulate the expression of functional gene product from the siaD gene (i.e. downregulating α-2-8 polysialyltransferase activity), preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. Such an inactivation is described in WO 01/09350. The siaD (also known as synD) mutation is the most advantageous of many mutations that can result in removing the human-similar epitope from the capsular polysaccharide, because it one of the only mutations that has no effect on the biosynthesis of the protective epitopes of LOS, thus being advantageous in a process which aims at ultimately using LOS as a protective antigen, and has a minimal effect on the growth of the bacterium. A preferred aspect of the invention is therefore a bleb immunogenic preparation as described above which is derived from an lgtE$^-$ siaD$^-$, an lgtA$^-$ siaD$^-$ or, preferably, an lgtB$^-$ siaD$^-$ meningococcus B mutant strain. The strain itself is a further aspect of the invention.

Although siaD$^-$ mutation is preferable for the above reasons, other mutations which switch off meningococcus B capsular polysaccharide synthesis may be used. Thus bleb production strain can be genetically engineered to permanently downregulate the expression of functional gene product from one or more of the

following genes: ctrA, ctrB, ctrC, ctrD, synA (equivalent to synX and siaA), synB (equivalent to siaB) or synC (equivalent to siaC) genes, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. The lgtE$^-$ mutation may be combined with one or more of these mutations. Preferably the lgtB$^-$ mutation is combined with one or more of these mutations. A further aspect of the invention is therefore a bleb immunogenic preparation as described above which is derived from such a combined mutant strain of meningococcus B. The strain itself is a further aspect of the invention.

A Neisserial locus containing various *lgt* genes, including lgtB and lgtE, and its sequence is known in the art (see M. P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021 and references cited therein, and J. Exp. Med. 180:2181-2190 [1994]).

Where full-length (non-truncated) LOS is to be used in the final product, it is desirable for LOS not to be sialyated (as such LOS generates an immune response against the most dangerous, invasive meningococcal B strains which are also unsialylated). In such case using a capsule negative strain which has a deleted synA (equivalent to synX and siaA), synB (equivalent to siaB) or synC (equivalent to siaC) gene is advantageous, as such a mutation also renders menB LOS incapable of being sialylated.

The Toxicity of LOS

The above purifed LOS or bleb immunogenic compositions of the invention may also be rendered less toxic by downregulating expression of certain genes in the bacterial production strain from which they are derived. Although such detoxification may not necessary for intranasal immunization with native OMV (J.J. Drabick *et al*, Vaccine (2000), **18**, 160-172), for parenteral vaccination detoxification would present an advantage. Preferably the LOS purifed LOS or bleb immunogenic compositions of the invention are detoxified by genetically engineering the Neisserial production strain by mutation/modification/inactivation of the genes involved in LipidA biosynthesis, particularly those genes involved in adding secondary acyl chains to lipidA, in particular by downregulating the expression of functional gene product from the msbB and/or htrB genes, and preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

Alternatively (or in addition) the purifed LOS or bleb immunogenic compositions can be derived from a Neisserial strain which has been genetically modified so that one or more of the following genes are upregulated (by introducing a stronger promoter or integrating an extra copy of the gene): pmrA, pmrB, pmrE and pmrF. Alternatively (or in addition) the purifed LOS or bleb immunogenic compositions may be detoxified by adding non-toxic peptide functional equivalents of polymyxin B [a molecule with high affinity for Lipid A] to the compositions. See WO 01/09350 for more detail on the above detoxification methods, and for relevant promoter / gene sequences and upregulation and downregulation methods. See WO 93/14115, WO 95/03327, Velucchi et al (1997) J Endotoxin Res 4: 1-12, and EP 976402 for further details of non-toxic peptide functional equivalents of polymyxin B that may be used in the compositions of this invention – particularly the use of the peptide SAEP 2 (of sequence KTKCKFLKKC where the 2 cysteines form a disulphide bridge).

By "downregulating the expression of functional gene product" it is meant that additions, deletions or additions are made to the promoter or open reading frame of the gene in questin such that the biosynthetic activity of the total gene product reduces (by 60, 70, 80, 90, 95 or most preferably 100%). Clearly frameshift mutations may be introduced, or weaker promoters substituted , however most preferably most or all of the open reading frame and/or promoter is deleted to ensure a permanent downregulation of the gene product (as described in WO 01/09350).

Further aspects of the invention include the above described genetically modified Neisserial (preferably meningococcal or gonococcal or meningococcal B) strains from which the LOS or bleb immunogenic preparations of the invention may be derived.

The LOS or LOS-containing bleb preparations of the invention

A further aspect of the invention are LOS preparations (particularly those described above) isolated from the Neisserial strains of the invention. Preferably the isolated LOS (or LOS-containing bleb) is L2 or L3 immunotype, and preferably the immunogenic compositions of the invention comprise both L2 and L3 LOS (or bleb) preparations of the invention.

Such preparations may also be improved by conjugating the oligosaccharide portion of the above LOS (whether purified or present in a bleb preparation) to a carrier comprising a source of T-cell epitopes (thus rendering the LOS an even better [T-dependent] immunogen). A purified LOS preparation of the invention may alternatively (or in addition) be rendered a better antigen by presenting it in liposome formulations known in the art (see for instance WO 96/40063 and references cited therein).

The process of isolation of LOS from bacteria is well known in the art (see for instance the hot water-phenol procedure of Wesphal & Jann [Meth. Carbo. Chem. 1965, 5:83-91]). See also Galanos et al. 1969, Eur J Biochem 9:245-249, and Wu et al. 1987, Anal Bio Chem 160:281-289. Techniques for conjugating isolated LOS are also known (see for instance EP 941738 incorporated by reference herein).

For the purposes of this invention "a carrier comprising a source of T-cell epitopes" is usually a peptide or, preferably, a polypeptide or protein. Conjugation techniques are well known in the art. Typical carriers include protein D from non typeable *H. influenzae*, tetanus toxoid, diphtheria toxoid, and CRM197.

Preferred isolated LOS compositions of the invention are: a composition comprising L2 and L3 isolated LOS wherein the oligosaccharide portion of each LOS is conjugated to a carrier comprising a source of T-cell epitopes, a composition comprising LOS which has a structure consistent with it having been derived from a lgtB$^-$ meningococcal strain wherein the oligosaccharide portion of each LOS is conjugated to a carrier comprising a source of T-cell epitopes, and most preferably a composition comprising L2 and L3 isolated LOS which have a structure consistent with them having been derived from an lgtB$^-$ meningococcal strain, wherein the oligosaccharide portion of each LOS is conjugated to a carrier comprising a source of T-cell epitopes. Preferably these compositions have been detoxified. This may be done by known techniques of hydrazine or alkaline hydrolysis chemical treatments which remove acyl chains from the molecule (but which may reduce the protective efficacy of the molecule), but is preferably done by isolating the LOS from an htrB$^-$ or msbB$^-$ meningococcal mutant (as described above), or by adding a non-toxic peptide functional equivalent of polymyxin B [a molecule with high affinity to Lipid A] to the composition, in particular SAEP 2 (as described above).

The LOS of the invention may be administered in an isolated state (usually in the form of micelles if the lipid A moiety is still intact), or may be administered in a liposome. In such case outer membrane proteins may be added to the liposome, and the LOS may be conjugated intra-liposome to such outer membrane proteins to render the oligosaccharide a T-dependent antigen. This may be done with a similar chemistry as described for intra-bleb LOS cross-linking as described below.

Intra-bleb cross-linking (conjugation) of the oligosaccharide portion of LOS to outer membrane proteins present on the surface of the bleb

Where LOS (in particular the LOS of the invention) is present in a bleb formulation the LOS is preferably conjugated in situ by methods allowing the conjugation of LOS to one or more outer membrane proteins also present on the bleb preparation (e.g. PorA or PorB in meningococcus).

This process can advantageously enhance the stability and/or immunogenicity (providing T-cell help) and/or antigenicity of the LOS antigen within the bleb formulation – thus giving T-cell help for the T-independent oligosaccharide immunogen in its most protective conformation – as LOS in its natural environment on the surface of meningococcal outer membrane. In addition, conjugation of the LOS within the bleb can result in a detoxification of the LOS (the Lipid A portion being stably buried in the outer membrane thus being less available to cause toxcity). Thus the detoxification methods mentioned above of isolating blebs from htrB$^-$ or msbB$^-$ mutants, or by adding non toxic peptide functional equivalent of polymyxin B to the composition may not be required (but which may be added in combination for additional security). Thus the inventors have found that a composition comprising blebs wherein LOS present in the blebs has been conjugated in an intra-bleb fashion to outer membrane proteins also present in the bleb can form the basis of a vaccine for the treatment or prevention of diseases caused by the organism from which the blebs have been derived, wherein such vaccine is substantially non-toxic and is capable of inducing a T-dependent bactericidal response against LOS in its native environment.

This invention therefore further provides such an intra-bleb LOS conjugated bleb preparation. Preferably the blebs have been derived from any Gram negative organism from which blebs can be produced (see WO 01/09350), preferably

Moraxella catarrhalis, non-typeable Haemophilus influenzae or Neisseria (most preferably meningococcus).

Such bleb preparations may be isolated from the bacterial in question (see WO 01/09350), and then subjected to known conjugation chemistries to link groups (e.g. NH_2 or COOH) on the oligosaccharide portion of LOS to groups (e.g. NH_2 or COOH) on bleb outer membrane proteins. Cross-linking techniques using glutaraldehyde, formaldehyde, or glutaraldehyde/formaldehyde mixes may be used, but it is preferred that more selective chemistries are used such as EDAC or EDAC/NHS (J.V. Staros, R.W. Wright and D. M. Swingle. Enhancement by N-hydroxysuccinimide of water-soluble carbodiimide-mediated coupling reactions. Analytical chemistry 156: 220-222 (1986); and Bioconjugates Techniques. Greg T. Hermanson (1996) pp173-176). Other conjugation chemistries or treatments capable of creating covalent links between LOS and protein molecules that could be used are described in EP 941738.

Preferably the bleb preparations are conjugated in the absence of capsular polysaccharide. The blebs may be isolated from a strain which does not produce capsular polysaccharide (naturally or via mutation), or may be purified from most and preferably all contaminating capsular polysaccharide. In this way, the intra-bleb LOS conjugation reaction is much more efficient.

Preferably more than 10, 20, 30, 40, 50, 60, 70, 80, 90, or 95% of the LOS present in the blebs is cross-linked/conjugated.

Where the intra-bleb conjugated blebs are derived from meningococcus, it is preferred that the strain from which they are derived is a mutant strain that cannot produce capsular polysaccharide (e.g. one of the mutant strains described above, in particular siaD$^-$). It is also preferred that immunogenic compositions effective against meningococcal disease comprise both an L2 and and L3 bleb, wherein the L2 and L3 LOS are both conjugated to bleb outer membrane proteins. Furthermore, it is preferred that the LOS structure within the intra-bleb conjugated bleb is consistent with it having been derived from an lgtB$^-$ meningococcal strain. Most preferably immunogenic compositions comprise intrableb-conjugated blebs: derived from a mutant meningococcal strain that cannot produce capsular polysaccharide and is lgtB$^-$; comprising L2 and L3 blebs derived from mutant meningococcal strains that cannot produce capsular polysaccharide; comprising L2 and L3 blebs derived from mutant meningococcal strains that are lgtB$^-$; or most preferably comprising L2 and L3 blebs

derived from mutant meningococcal strains that cannot produce capsular polysaccharide and are lgtB$^-$.

Typical L3 meningococcal strain that can be used for the present invention is H44/76 menB strain. A typical L2 strain is the B16B6 menB strain or the 39E meningococcus type C strain.

As stated above, the blebs of the invention have been detoxified to a degree by the act of conjugation, and need not be detoxified any further, however further detoxification methods may be used for additional security, for instance using blebs derived from a meningococcal strain that is htrB$^-$ or msbB$^-$ or adding a non-toxic peptide functional equivalent of polymyxin B [a molecule with high affinity to Lipid A] (preferably SEAP 2) to the bleb composition (as described above).

In the above way meningococcal blebs and immunogenic compositions comprising blebs are provided which have as an important antigen LOS which is substantially non-toxic, devoid of autoimmunity problems, has a T-dependent character, is present in its natural environment, and is capable of inducing a bactericidal antibody response against more than 90% of meningococcal strains (in the case of L2+L3 compositions).

One or more of Men A, C, Y or W capsular polysaccharides or oligosaccharides (preferably at least MenC, MenA and MenC, or Men C and MenY) may also be conjugated onto an outermembrane protein of the bleb of the invention as well. Although this could be done in the same reaction as LOS cross-linking, it is preferred that this is done in a separate reaction.

The process of optimal intra-bleb LOS conjugation is a further aspect of the present invention.

Intrableb conjugation should incorporate 1, 2 or all 3 of the following process steps: conjugation pH should be greater than pH 7.0, preferably greater than or equal to pH 7.5 (most preferably under pH 9); conditions of 1-5% preferably 2-4% mostpreferably around 3% sucrose should be maintained during the reaction; NaCl should be minimised in the conjugation reaction, preferably under 0.1M, 0.05M, 0.01M, 0.005M, 0.001M, and most preferably not present at all. All these process features make sure that the blebs remain stable and in solution throughout the conjugation process.

The EDAC/NHS conjugation process is a preferred process for intra-bleb conjugation. EDAC/NHS is preferred to formalydehyde which can cross-link to too high an extent thus adversely affecting filterability. EDAC reacts with carboxylic acids to create an active-ester intermediate. In the presence of an amine nucleophile, an amide bond is formed with release of an isourea by-product. However, the efficiency of an EDAC-mediated reaction may be increased through the formation of a Sulfo-NHS ester intermediate. The Sulfo-NHS ester survives in aqueous solution longer than the active ester formed from the reaction of EDAC alone with a carboxylate. Thus, higher yields of amide bond formation may be realized using this two-stage process. EDAC/NHS conjugation is discussed in J.V. Staros, R.W. Wright and D. M. Swingle. Enhancement by N-hydroxysuccinimide of water-soluble carbodiimide-mediated coupling reactions. Analytical chemistry 156: 220-222 (1986); and Bioconjugates Techniques. Greg T. Hermanson (1996) pp173-176.

A preferred process of the invention is therefore a process for producing intra-bleb conjugated LOS (preferably meningococcal) comprising the steps of conjugating blebs in the presence of EDAC/NHS at a pH between pH 7.0 and pH 9.0 (preferably around pH 7.5), in 1-5% (preferably around 3%) sucrose, and optionally in conditions substantially devoid of NaCl (as described above), and isolating the conjugated blebs from the reaction mix.

The reaction may be followed on separation gels of the reaction mixture using anti-LOS (e.g. anti-L2 or anti-L3) mAbs to show the increase of LOS molecular weight for a greater proportion of the LOS in the blebs as reaction time goes on.

Yields of 99% blebs can be recovered using such techniques.

EDAC was found to be an excellent intra-bleb cross-linking agent in that it cross-linked LOS to OMP sufficiently for improved LOS T-dependent immunogenicity, but did not cross link it to such a high degree that problems such as poor filterability and inter-bleb cross-linking occurred. A too high cross-linking should also avoided to avoid any decrease in immunogenicity of protective OMPs naturally present on the surface of the bleb e.g. TbpA.

Techniques for isolating blebs

Outer Membrane Vesicles (OMVs or blebs) can be isolated by many known techniques (Fredriksen *et al*, NIPH Annals (1991), **14**, 67-79; Zollinger *et al*, J. Clin

Invest (1979), **63**, 836-848; Saunders *et al*, Infect Immun (1999), **67**, 113-119; J.J. Drabick *et al*, Vaccine (1999), **18**, 160-172). These divide into 2 main groups – techniques which use deoxycholate (about 0.5%) to extract blebs from meningococcus, and techniques that use low levels of deoxycholate (DOC) or no deoxycholate at all. DOC free process blebs have the interesting feature of maintaining high level of LOS in the OMV – which is advantageous in a vaccine where LOS is a protective antigen. Compared to DOC extracted blebs, the concentration of L3 Ags in OMV obtained by a DOC free process is approximately ten times higher. A detergent-free (preferably DOC-free) process of preparing blebs is preferred for the purposes of the processes of this invention for this reason, although extraction with a buffer containing low levels of detergent (preferably DOC) may also be advantageous in that the step would leave most of the tightly interacting LOS in the bleb whilst removing any more toxic loosely retained LOS. Preferably 0-0.3% detergent (preferably DOC) is used for bleb extraction, more preferably 0.05-0.2%, and most preferably around 0.1%. DOC free (or low DOC) extraction processes are particularly preferred where the LOS has been detoxified by one of the methods detailed above.

Vaccine Compositions

The immunogenic compositions of the invention may readily be formulated as vaccine compositions by adding a pharmaceutically acceptable excipient.

A process for making the Neisserial (preferably meningococcal) immunogenic compositions or vaccines of the invention is further provided comprising the steps of isolating, purified LOS of the invention (preferably L2 or L3) as described above or producing isolated blebs of the invention (preferably with an L2 or L3 immunotype) as described above, and formulating the LOS or blebs with a pharmaceutically acceptable excipient. Preferably purified LOS of both immunotype L2 and L3 of the invention, or blebs of both immunotype L2 and L3 of the invention, or a purifed LOS of L2 and a bleb of L3 (or vice versa), are combined in a mixing step. Preferably the purified LOS or bleb of the invention has been conjugated as decribed above after isolation. An additional liposome formulation step may also be added for the purified LOS (using techniques known in the art - see for instance WO 96/40063 and

references cited therein). Preferably bleb preparations are isolated by extraction with low (or no) concentrations of DOC (as described above).

Such L2 and L3 combination processes can yield a vaccine which is effective against almost all meningococcal B strains.

The above immunogenic compositions (or processes) may have added one or more (2, 3 or 4) meningococcal polysaccharides (either plain or conjugated to a carrier comprising T-cell epitopes) from serogroups A, C, Y or W to the composition. Preferably at least C is added (most preferably conjugated), and more preferably A and C or Y and C (preferably all conjugated) and most preferably A, C, Y and W (preferably all conjugated). The term "polysaccharide" is intended to cover unsized or sized (reduced in size) polysaccharides, or sized oligosaccharides. Advantageously a conjugated H. influenzae B capsular polysaccharide is also included in the above compositions to generate a universal meningitis vaccine.

Vaccine Formulations of the invention

The immunogenic compositions of the invention may be formulated with a suitable adjuvant to generate vaccine compositions of the invention.

Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate (preferably aluminium hydroxide), but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable Th1 adjuvant systems that may be added include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A (or other non-toxic derivatives of LPS), and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) [or non toxic LPS derivatives] together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 [or other saponin] and 3D-MPL [or non toxic LPS derivative] as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 [or saponin] is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation that may be

added. Other adjuvants that may be added comprise a saponin, more preferably QS21 and/or an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) may also be added

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

An immunoprotective dose of vaccines can be administered via the systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary (preferably intra-nasal administration), respiratory, genitourinary tracts. Typically bleb quantity in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100μg of each bleb, preferably 5-50μg, and most typically in the range 5 - 25μg.

Further improvements to the bleb immunogenic compositions of the invention

The above bleb compositions of the invention may be further improved in eficacy in vaccines of the invention if the Neisserial strain from which they are derived (including gonococcus, and preferably meningococcus, most preferably *N. meningitidis* B) have one or more of the following genes (encoding protective antigens) upregulated by inserting further copies of the gene into the genome, or introducing a stronger promoter upstream of the existing gene, or any of the other ways discussed in WO 01/09350 which are capable of inducing modified strains to make over 1.2, 1.5, 2, 3, 5 or 10 times the level of antigen as compared to the unmodified strain: NspA (WO 96/29412), Hsf-like (WO 99/31132), Hap (PCT/EP99/02766), OMP85 (WO 00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO 96/31618), TbpA (WO92/03467, US5912336, WO93/06861 and EP586266), TbpB (WO93/06861 and EP586266), NadA (Comanducci et al J. Exp. Med. 2002 195; 1445-1454), FrpA/FrpC or portions in common between these antigens involving 5 or more repeat sequences (WO

92/01460; Thompson *et al.*, (1993) J. Bacteriol. 175:811-818; Thompson *et al.*, (1993) Infect. Immun.. 61:2906-2911), LbpA, LbpB (PCT/EP98/05117), FhaB (WO98/02547 SEQ ID NO 38 [nucleotides 3083-9025]), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), Tbp2 (WO 99/57280), MltA (WO 99/57280), TspA (WO 00/03003), TspB (WO 00/03003) and ctrA (PCT/EP00/00135). It is particularly preferred if both Hsf and TbpA (Low or High, or both Low and High molecular weight forms [EP 586266]), or Hsf and OMP85, or OMP85 and TbpA (Low or High, or both Low and High molecular weight forms), or NspA and Hsf, or NspA and OMP85, or NspA and TbpA (Low or High, or both Low and High molecular weight forms) are both upregulated. TbpA may be upregulated genetically or by growing the meningococcal production strains in iron limited conditions for instance in the presence of 50-70 μM Desferal. If latter approach is taken, it is preferred that the FrpB gene expression is downregulated (preferably deleted) as this variable antigen may become immunodominant in blebs isolated from meningococcal strains isolated in Iron limited conditions.

Ghost or Killed Whole cell vaccines

The inventors envisage that the above compositions and vaccines concerning blebs can be easily extended to processes concerning ghost or killed whole cell preparations and vaccines (with identical advantages). Methods of making ghost preparations (empty cells with intact envelopes) from Gram-negative strains are well known in the art (see for example WO 92/01791). Methods of killing whole cells to make inactivated cell preparations for use in vaccines are also well known. Therefore the compositions and vaccines involving blebs described throughout this document are envisioned to be applicable to the the same compositions or vaccines comprising equivalent ghost and killed whole cell preparations of the invention.

Serum bactericidal assay

The serum bactericidal assay is the preferred method to assess synergistic relationships between antigens when combined in an immunogenic composition of the invention.

Such a synergistic response may be characterised by the SBA elicited by the combination of antigens being at least 50%, two times, three times, preferably four times, five times, six times, seven times, eight times, nine times and most preferably ten times higher than the SBA elicited by each antigen separately. Preferably SBA is measured against a homologous strain from which the antigens are derived and preferably also against a panel of heterologous strains. (See below for a representative panel for instance BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16)). SBA is the most commonly agreed immunological marker to estimate the efficacy of a meningococcal vaccine (Perkins et al. J Infect Dis. 1998, 177:683-691). Satisfactory SBA can be acertained by any known method. SBA can be carried out using sera obtained from animal models, or from human subjects.

A preferred method of conducting SBA with human sera is the following. A blood sample is taken prior to the first vaccination, two months after the second vaccination and one month after the third vaccination (three vaccinations in one year being a typical human primary vaccination schedule administered at, for instance, 0, 2 and 4 months, or 0, 1 and 6 months). Such human primary vaccination schedules can be carried out on infants under 1 year old (for instance at the same time as Hib vaccinations are carried out) or 2-4 year olds or adolescents may also be vaccinated to test SBA with such a primary vaccination schedule. A further blood sample may be taken 6 to 12 months after primary vaccination and one month after a booster dose, if applicable.

SBA will be satisfactory for an antigen or bleb preparation with homologous bactericidal activity if one month after the third vaccine dose (of the primary vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against the strain of meningococcus from which the antigens of the invention were derived is greater than 30%, preferably greater than 40%, more preferably greater than 50%, and most preferably greater than 60% of the subjects.

Of course an antigen or bleb preparation with heterologous bactericidal activity can also constitute bleb preparation with homologous bactericidal activity if it

can also elicit satisfactory SBA against the meningococcal strain from which it is derived.

SBA will be satisfactory for an antigen or bleb preparation with heterologous bactericidal activity if one month after the third vaccine dose (of the primary vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against three heterologous strains of meningococcus is greater than 20%, preferably greater than 30%, more preferably greater than 35%, and most preferably greater than 40% of the subjects. Such a test is a good indication of whether the antigen or bleb preparation with heterologous bactericidal activity can induce cross-bactericidal antibodies against various meningococcal strains. The three heterologous strains should preferably have different electrophoretic type (ET)-complex or multilocus sequence typing (MLST) pattern (see Maiden et al. PNAS USA 1998, 95:3140-5) to each other and preferably to the strain from which the antigen or bleb preparation with heterologous bactericidal activity is made or derived. A skilled person will readily be able to determine three strains with different ET-complex which reflect the genetic diversity observed amongst meningococci, particularly amongst meningococcus type B strains that are recognised as being the cause of significant disease burden and/or that represent recognised MenB hyper-virulent lineages (see Maiden et al. *supra*). For instance three strains that could be used are the following: BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16) belonging to the ET-5 complex, or any other strains belonging to the same ET/Cluster. Such strains may be used for testing an antigen or bleb preparation with heterologous bactericidal activity made or derived from, for instance, meningococcal strain CU385 (B:4:P1.15) which belongs to the ET-5 complex. Another sample strain that could be used is from the Lineage 3 epidemic clone (e.g. NZ124 [B:4:P1.7,4]). Another ET-37 strain is NGP165 (B:2a:P1.2).

Processes for measuring SBA activity are known in the art. For instance a method that might be used is described in WO 99/09176 in Example 10C. In general terms, a culture of the strain to be tested is grown (preferably in conditions of iron depletion – by addition of an iron chelator such as EDDA to the growth medium) in the log phase of growth. This can be suspended in a medium with BSA (such as

Hanks medium with 0.3% BSA) in order to obtain a working cell suspension adjusted to approximately 20000 CFU/ml. A series of reaction mixes can be made mixing a series of two-fold dilutions of sera to be tested (preferably heat-inactivated at 56°C for 30 min) [for example in a 50µl/well volume] and the 20000 CFU/ml meningococcal strain suspension to be tested [for example in a 25µl/well volume]. The reaction vials should be incubated (e.g. 37°C for 15 minutes) and shaken (e.g. at 210 rpm). The final reaction mixture [for example in a 100µl volume] additionally contains a complement source [such as 25 % final volume of pretested baby rabbit serum], and is incubated as above [e.g. 37°C for 60 min]. A sterile polystyrene U-bottom 96-well microtiter plate can be used for this assay. A aliquot [e.g. 10 µl] can be taken from each well using a multichannel pipette, and dropped onto Mueller-Hinton agar plates (preferably containing 1 % Isovitalex and 1 % heat-inactivated Horse Serum) and incubated (for example for 18 hours at 37°C in 5 % CO_2). Preferably, individual colonies can be counted up to 80 CFU per aliquot. The following three test samples can be used as controls: buffer + bacteria + complement; buffer + bacteria + inactivated complement; serum + bacteria + inactivated complement. SBA titers can be straightforwardly calculated using a program which processes the data to give a measurement of the dilution which corresponds to 50 % of cell killing by a regression calculation.

All references or patent applications cited within this patent specification are incorporated by reference herein.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1:

Examples describing deletions genes encoding proteins involved in B capsular polysaccharide production of meningococcus B, the deletion of the PorA gene, the upregulation of various protective outer membrane proteins on the surface of meningococcal blebs, the downregulation of immunodominant proteins or biosynthetic enzymes, and processes for isolating blebs are described in WO 01/09350.

Example 2: Experiments with L3 and intermediate (lgtB⁻) DOC free blebs (non-detoxified LOS) induced cross-bactericidal antibodies

The MC58 meningococcal derivative strain used is B:P1.7.16, *opc-*, *siaD-*. This strain was genetically modified to express either L3 (strain 2G2) or an intermediate epitope (strain 2G EcoN1b-1), as 2G2 but *lgtB* additionally turned off) or an LPS in short version (strain C6, *lgtE* off). OMV were produced according either a normal high DOC process or DOC free process.

Mice (10 per group) were immunized three times by the intra-muscular route on Day 0, 20 and 28. They received 1 or 10 μg (protein content) of blebs formulated on Al(OH)3. Blood samples were taken on day 28 (post II) and day 42 (post III).

Bactericidal assays were done on pooled sera and using homologous strains (MC58 and H44/76) and two heterologous strains (M97250687 and M9725078) with baby rabbit serum as source of exogenous complement.

The following table summarizes the results (bactericidal titers for 50% killing):

Antigen	Blood samples	Strain and serotype: MC58 P1.7.16	H44/76TT P1.7.16	M97250687 P1.19.15	M97252078 P1.4
c6 no doc 10ug IM	Post II	>2560	>2560	>2560	98
c6 no doc 10ug IM	Post III	1 353	>2560	>2560	90
c6 no doc 1ug IM	Post II	247	620	247	<20
c6 no doc 1ug IM	Post III	411	878	748	<20
2g2 no doc 10ug IM	Post II	>320	>2560	>2560	>2560
2g2 no doc 10ug IM	Post III	>2560	>2560	>2560	1407
2g2 no doc 1ug IM	Post II	>2560	>2560	>2560	119
2g2 no doc 1ug IM	Post III	>2560	>2560	>2560	348
2gecoN1b-1 no doc 10ug IM	Post II	>2560	>2560	>2560	1162
2gecoN1b-1 no doc 10ug IM	Post III	>2560	>2560	>2560	1213
2gecoN1b-1 no doc 1ug IM	Post II	1 151	>2560	1 696	22
2gecoN1b-1 no doc 1ug IM	Post III	2 220	>2560	1 947	135
c6 doc 10ug IM	Post II	308	248	341	<20
c6 doc 10ug IM	Post III	189	104	400	<20
c6 doc 1ug IM	Post II	33	43	63	<20
c6 doc 1ug IM	Post III	NC (>20)	24	156	<20
2g2 doc 10ug IM	Post II	NC (>20)	25	360	<20
2g2 doc 10ug IM	Post III	201	<20	647	<20
2g2 doc 1ug IM	Post II	275	<20	299/644	<20
2g2 doc 1ug IM	Post III	237	<20	728	<20
2gecoN1b-1 doc 10ug IM	Post II	573	31	685	<20
2gecoN1b-1 doc 10ug IM	Post III	NC (>40)	21	1 140	<20
2gecoN1b-1 doc 1ug IM	Post II	261	NC	118	<20
2gecoN1b-1 doc 1ug IM	Post III	348	NC	692	<20

Clearly, the presence of L3 (2g2) or intermediate (2gecon1b-1) epitope induces cross-bactericidal antibodies, while blebs from truncated LPS strain (C6) induce lower level of cross-reacting antibodies. This was particularly illustrated when 1µg of OMV was injected.

Moreover, as shown with OMV purified with DOC, reducing the LPS content of blebs reduces the induction of cross-bactericidal antibodies. Aside from increased LPS, it is possible that DOC free blebs may also advantageously retain some proteins losely interacting with the OMVs such as lipoproteins.

Example 3: Intra-bleb cross-linking of L3 LOS and Outer-Membrane Protein

The MenB blebs used were derived from an H44/76 strain (LOS immunotype L3) that was SiaD$^-$ (thus not expressing capsular polysaccharide) and PorA$^-$. Two different strains were used: a full L3 (strain B1717, siad(-) PorA (-) Full L3) and a truncated L3 (strain B1727, siad(-) PorA (-) lgtB(-) TrL3).

The EDAC /NHS conjugation process was used according to known methods to cross-link LOS and OMP within the blebs to render the oligosaccharide component of LOS a T-dependent antigen (EDAC /NHS was preferred to formalydehyde which was found to cross-link to too high an extent thus adversely affecting filterability). EDAC reacts with carboxylic acids to create an active-ester intermediate. In the presence of an amine nucleophile, an amide bond is formed with release of an isourea by-product. However, the efficiency of an EDAC-mediated reaction may be increased through the formation of a Sulfo-NHS ester intermediate. The Sulfo-NHS ester survives in aqueous solution longer than the active ester formed from the reaction of EDAC alone with a carboxylate. Thus, higher yields of amide bond formation may be realized using this two-stage process. EDAC/NHS conjugation is discussed in J.V. Staros, R.W. Wright and D. M. Swingle. Enhancement by N-hydroxysuccinimide of water-soluble carbodiimide-mediated coupling reactions. Analytical chemistry 156: 220-222 (1986); and Bioconjugates Techniques. Greg T. Hermanson (1996) pp173-176.

The reaction mixture contained 1.5 mg Sulfo-NHS and 5 mg EDAC in 3% sucrose (for bleb stability) in a volume of 1mL. Blebs were present in a ratio of 0.025mg EDAC / mg blebs. Blebs were present in a concentration of 2 mg/ml and pH was adjusted to 7.5 with HCl 0.1 N.

The reaction was left for 4 hours at room temperature, and the mixture was dialysed against 2 mM phosphate buffer containing 3% sucrose, pH 7.5. The mixture was then filtered on Sterivex G10 0.22 μm. A yield of 99% blebs was recovered.

EDAC was found to be an excellent intra-bleb cross-linking agent in that it cross-linked LOS to OMP sufficiently for improved LOS T-dependent immunogenicity, but did not cross link it to such a high degree that problems such as poor filterability and inter-bleb cross-linking occurred. A too high cross-linking should also avoided to avoid any decrease in immunogenicity of protective OMPs naturally present on the surface of the bleb e.g. TbpA.

We Claim:

1. A Neisserial bleb preparation derived from a neisserial strain which has had 2 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.

2. A Neisserial bleb preparation derived from a neisserial strain with an L2 LOS immunotype which has had 1 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.

3. A Neisserial bleb preparation derived from a neisserial strain with an L3 LOS immunotype which has had 1 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.

4. A multivalent Neisserial bleb preparation derived from a neisserial strain with an L2 LOS immunotype and a neisserial strain with an L3 LOS immunotype, wherein one or more of said strains has had 1 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.

5. A meningococcal serogroup B bleb preparation derived from a meningococcal serogroup B strain which has had either or both of the following genes downregulated in expression, and preferably deleted, compared to the native strain: siaD [or, alternatively, ctrA, ctrB, ctrC, ctrD; synA (equivalent to synX and siaA), synB (equivalent to siaB) or synC (equivalent to siaC)] and lgtB.

6. The Neisserial bleb preparation of claims 1-3 or the multivalent Neisserial bleb preparation of claim 4 derived from meningococcal serogroup B strains as claimed in claim 5.

7. The Neisserial bleb preparation or the multivalent Neisserial bleb preparation of any one of claims 1-6 derived from neisserial strains which have had any of the following combinations of outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strains: PorA and OpA, PorA and OpC, OpA and OpC, PorA and OpA and OpC.

8. The Neisserial bleb preparation or the multivalent Neisserial bleb preparation of any one of claims 1-7 derived from detoxified neisserial strains which have had either or both of the following combinations of genes downregulated in expression, and preferably deleted, compared to the native strains: msbB or htrB.

9. The Neisserial strain from which the Neisserial bleb preparations of claims 1-8 are derived.

10. A LOS preparation isolated from the Neisserial strain of claim 9.

11. The LOS preparation of claim 10 comprising immunotype L2 and L3 LOS.

12. The LOS preparation of claim 10 or 11 in a liposome formulation.

13. The Neisserial bleb preparation of any one of claims 1-8 or the LOS preparation of claims 10-12, wherein the LOS contained therein is conjugated to a source of T-helper epitopes, preferably a protein or outer membrane protein.

14. An immunogenic composition or vaccine comprising the Neisserial bleb preparation or the LOS preparation of any one of claims 1-8 or 10-13, and a pharmaceutically acceptable excipient.

15. The vaccine of claim 14, additionally comprising an adjuvant, preferably aluminium hydroxide.

16. The vaccine of claim 14 or 15 additionally comprising one or more conjugated capsular polysaccarides or oligosaccharides derived from the following strains:

meningococcus serogroup A, meningococcus serogroup C, meningococcus serogroup W-135, meningococcus serogroup Y, and *H. influenzae* type b.

17. A process of manufacturing the Neisserial bleb preparation vaccine of claim 14 comprising the steps of culturing a Neisserial strain of claim 9, isolating blebs therefrom, and formulating the blebs with a pharmaceutically acceptable excipient.

18. The process of claim 17, wherein the isolation step is carried out by extracting with 0-0.3% deoxycholate.

19. A bleb preparation from a Gram-negative bacterial strain in the outer-membrane of which is integrated an outer-membrane protein conjugated to LOS.

20. The bleb preparation of claim 19, wherein more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 or 99% of the conjugated LOS has its lipid A moiety integrated in the outer-membrane of the bleb and/or in an environment whereby its toxicity is reduced or shielded from a host to which it has been administered.

21. The bleb preparation of claim 19 or 20, wherein more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 or 99% of the conjugated LOS is in a native conformation suitable for inducing a bactericidal antibody response against it when administered to a host's immune system.

22. The bleb preparation of claims 19-21, wherein the outer-membrane protein is conjugated to the oligosaccharide or polysaccharide moiety of the LOS molecule.

23. The bleb preparation of claim 19-22, wherein the outer-membrane protein and LOS molecule are native to the Gram-negative bacterial strain from which the blebs are derived.

24. The bleb preparation of claims 19-24 obtainable by a process of intra-bleb cross-linking.

25. The bleb preparation of claims 19-24 wherein more than 10, 20, 30, 40, 50, 60, 70, 80, 90, or 95% of the LOS present in the blebs is cross-linked.

26. The bleb preparation of claims 19-25 derived from a Gram-negative strain that does not produce capsular polysaccharide.

27. The bleb preparation of claims 19-26, wherein capsular polysaccharide is not conjugated to a outer-membrane protein integrated in the bleb preparation.

28. The bleb preparation of claims 19-27 derived from a *Moraxella catarrhalis* or a non-typeable *Haemophilus influenzae* strain.

29. The bleb preparation of claims 19-27 derived from a Neisserial strain, preferably *Neisseria meningitis*.

30. The bleb preparation of claim 29 wherein the LOS has a truncated structure consistent with it having been derived from a strain which is lgtB$^-$.

31. The bleb preparation of claim 29 or 30 wherein the bleb preparation comprises a mixture of blebs of L2 and L3 LOS immunotypes.

32. The bleb preparation of claims 29-31, wherein the LOS Lipid A moiety lacks secondary acyl chains consistent with it having been isolated from a htrB$^-$ or msbB$^-$ meningococcal strain.

33. An immunogenic composition or vaccine comprising the bleb preparation of claims 19-32, and a pharmaceutically acceptable excipient.

34. The immunogenic composition or vaccine of claim 33 additionally comprising an adjuvant, preferably aluminium hydroxide.

35. The immunogenic composition or vaccine of claim 33 or 34 additionally comprising one or more conjugated capsular polysaccarides or oligosaccharides

derived from the following strains: meningococcus serogroup A, meningococcus serogroup C, meningococcus serogroup W-135, meningococcus serogroup Y, and *H. influenzae* type b.

36. A process of producing a intra-bleb conjugated bleb preparation from a Gram-negative bacterial strain in the outer-membrane of which is integrated an outer-membrane protein conjugated to LOS, comprising the steps of:

a) isolating blebs from the Gram-negative strain,

b) carrying out chemistry suitable for conjugating the oligosaccharide moiety of the LOS present in the blebs to a outer membrane protein present on the same bleb,

c) isolating the intra-bleb conjugated bleb preparation, and

d) optionally formulating the intra-bleb conjugated bleb preparation with a further intra-bleb conjugated bleb preparation made by the same process but having a different LOS immunotype and/or formulating the bleb preparation with a pharmaceutically acceptable excipient to make a vaccine composition.

37. The process of claim 36 wherein in step a) the blebs are extracted using a low concentration, such as 0-0.3%, deoxycholate.

38. The process of claim 36 or 37, wherein in step b) the pH is kept between 7 and 9, preferably around pH 7.5.

39. The process of claims 36-38, wherein step b) is carried out in 1-5% sucrose, preferably around 3%.

40. The process of claims 36-39, wherein step b) is carried out in low NaCl concentration conditions.

41. The process of claims 36-40, wherein step b) is carried out with EDAC/NHS chemistry.

42. The process of claims 36-41, wherein in step a) the blebs are isolated from a meningococcal strain, preferably a meningococcus B strain.

43. The process of claim 42 wherein the meningococcal strain cannot make capsular polysaccharide, and is preferably a siaD$^-$ mutant.

44. The process of claim 42 or 43 wherein the meningococcal strain is an lgtB$^-$ mutant.

45. The process of claims 42-44 wherein the meningococcal strain has an L2 LOS immunotype.

46. The process of claims 42-44 wherein the meningococcal strain has an L3 LOS immunotype.

47. The process of claims 42-46 wherein in step d) a meningococcal intra-bleb conjugated bleb preparation with an L2 immunotype is combined with a further meningococcal intra-bleb conjugated bleb preparation with an L3 immunotype.

ABSTRACT

The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are more suitable for the production of neisserial, in particular meningococcal, outer-membrane vesicle (or bleb) vaccines. Advantageous processes and vaccine products are also described based on the use of novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines which have been rendered safer for use in human subjects. In particular combinations of gene downregulations are described such as PorA & OpA, PorA and OpC, OpA and OpC, and PorA and OpA and OpC. Additionally a combination of gene downregulations are described to render meningococcal B blebs safer: namely SiaD and lgtB.

Figure 1: L3 and L2 immunotypes (H44/76 , MC58 strains)

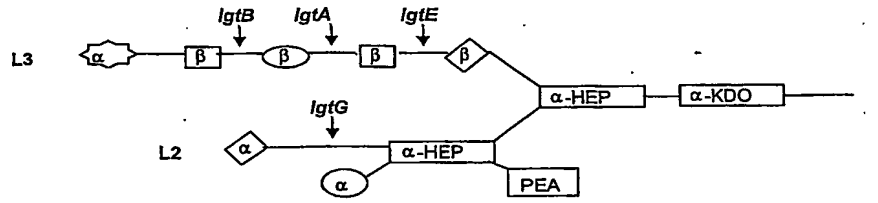

Figure 2: L3 and L1 immunotypes (e.g. 126E strain)